AD_____

Award Number: DAMD17-03-1-0644

TITLE: Characterization of Breast Cancer Stem Cells

PRINCIPAL INVESTIGATOR: David L. Crowe, Ph.D.

CONTRACTING ORGANIZATION: University of Southern California
Los Angeles, CA 90089-9074

REPORT DATE: August 2005

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-08-05	Final Addendum	16 Jul 04 - 15 Jul 05

4. TITLE AND SUBTITLE
Characterization of Breast Cancer Stem Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0644

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
David L. Crowe, Ph.D.

Email-dcrowe@usc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Southern California
Los Angeles, CA 90089-9074

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:

Abstract can be found on next page.

15. SUBJECT TERMS
biomarkers, cancer biology, endogenous factors

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	14	19b. TELEPHONE NUMBER *(include area code)* 301-619-7325

ABSTRACT

Cellular markers which identify putative breast epithelial stem cells have been reported. Certain clinical aspects of breast cancer could result from transformed stem cells in the malignant tumor. Breast cancer may therefore originate from neoplastic transformation of normal breast epithelial stem cells. These transformed stem cells exist in the tumor as rare cells with properties that drive aspects of tumorigenesis. This model predicts slower cell cycle progression, greater resistance to DNA damage, increased in vitro invasion, and in vivo tumorigenesis. We tested the hypothesis that transformed stem cells drive multiple aspects of breast tumorigenesis by functionally characterizing their biological properties. The hypothesis was tested by sorting of putative breast cancer stem cell populations followed by cell cycle analysis, in vitro and in vivo proliferation and invasion assays, and sensitivity to chemotherapy and radiation induced DNA damage. Putative cancer stem cells generally exhibited slower cell cycle progression, greater resistance to DNA damage, and increased tumor formation. However, the stem-like properties of these cells varied widely depending on the line from which they were isolated.

Table of Contents

INTRODUCTION

Stem cells have been the subject of great interest in recent years because of their unique biological properties: slow cell cycle progression, self renewal, and generation of differentiated cell types (Blau et al., 2001). Cellular markers which identify putative breast epithelial stem cells have been reported recently (Gudjonsson et al., 2002). Certain clinical aspects of the cancer could result from transformed stem cells residing within the malignant tumor (Reya et al., 2001). There is evidence that cancer stem cells exist in some hematologic malignancies but the case for transformed stem cells in breast cancer is less clear. Oncogenic mutations could be inherited from transformed epithelial stem cells and thus give rise to diverse tumorigenic populations observed in breast cancer. There are important ramifications for clinical cancer treatment if the growth of breast cancer is dependent on a subset of transformed stem cells. Tumor recurrence following traditional radiation and chemotherapy may be due to the slowly dividing and thus untargeted stem cell compartment repopulating the tumor. The current study hypothesized that breast originates from neoplastic transformation of normal breast epithelial stem cells. These transformed stem cells exist in the tumor as rare cells with properties which drive multiple aspects of tumorigenesis. We tested this hypothesis by functionally characterizing the biological properties of putative breast cancer stem cells. The following predictions of the model were tested: breast cancer stem cells infrequently enter the cell cycle, are resistant to anti-proliferative therapy, and are more tumorigenic in animal models.

BODY OF REPORT

This is the final report of Concept Award DAMD17-03-1-0644 "Characterization of Breast Cancer Stem Cells". The results of this study were presented at the Department of Defense Breast Cancer Research Program meeting in Philadelphia, PA in June 2005. Double immunofluorescence labeling and fluorescence activated cell sorting (FACS) of cultured human breast cancer cell lines using antibodies to sialomucin and epithelial specific antigen (ESA) revealed a predominant ESA+ population (>99%) in all lines examined (MCF7, T47D, MDA-MB-468, MDA-MB-231, Hs578T, and SKBR3). MCF7 and T47D contained a sialomucin negative population (MUC-) that was much smaller than other cell lines (0.05% and 0.02% respectively). In contrast, the sialomucin negative population in the other four lines was 0.4-0.5%. We also examined additional markers of breast epithelial cell differentiation (Fig. 1). Both populations in all cell lines expressed keratin 18, a marker of simple epithelium. In addition, all cell lines expressed keratin 19, which is expressed by the terminal duct lobular unit. Only SKBR3 expressed detectable levels of smooth muscle actin, a marker of myoepithelial differentiation. We detected phenotypic differences in the ESA+/MUC- population between different breast cancer cell lines. All ESA+/MUC- populations proliferated 10-20% slower than the MUC+ cells; however the MUC- fraction in MCF7 and T47D lines grew at 25-30% slower rates as determined by in vitro proliferation, DNA content analysis, and BrdU incorporation (Fig. 2A, B). We also determined differences in the relative abilities of these populations to invade Matrigel membranes. While the MUC- fraction in the other 4 cell lines were highly invasive in the in vitro assay, this subpopulation in MCF7 and T47D cells were 50-80% less able to penetrate the reconstituted basement membrane (Fig. 3). With respect to DNA damage by etoposide and ionizing radiation, the MUC- population from MCF7 and T47D cells showed 60% less DNA damage in the single cell gel electrophoresis analysis than the sorted fraction from the other 4 cell lines (Fig. 4). However, the MUC- population in these lines was more resistant to DNA damage than the MUC+ fraction. The MCF7 and T47D MUC- population were also 90% less tumorigenic (as measured by

tumor volume) in nude mice than the same fraction from the other 4 cell lines (Fig. 5). However, all MUC- populations were more tumorigenic than the MUC+ fractions.

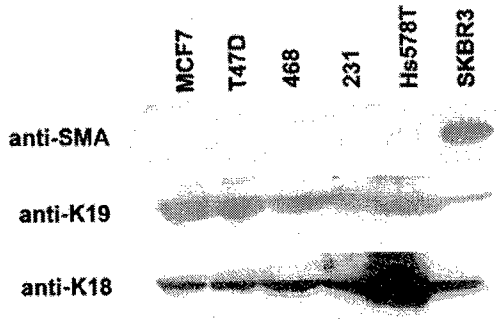
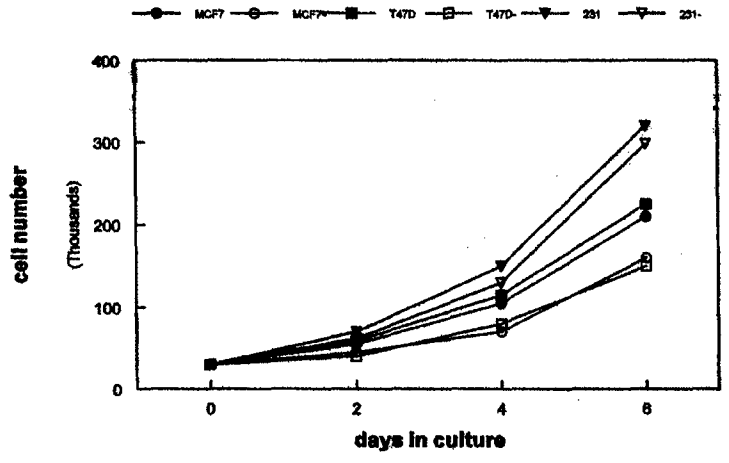


Fig. 1. Expression of differentiation markers in human breast cancer cell lines. Six breast cancer cell lines were examined by western blot using antibodies to the myoepithelial marker smooth muscle actin (SMA), the terminal ductal marker keratin 19 (K19), and the simple epithelial marker keratin 18 (K18). Representative blots are shown.

A



B

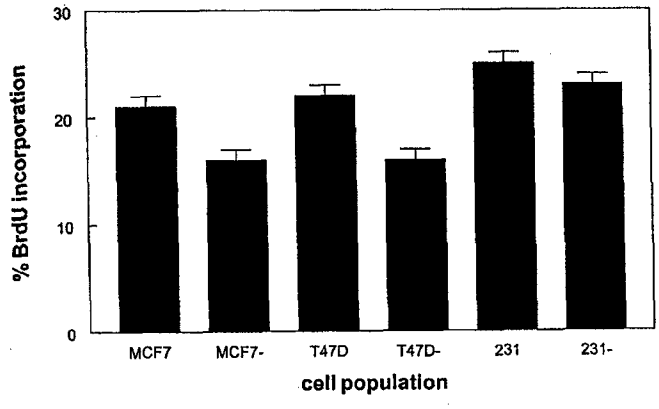


Fig. 2. Decreased proliferation of MUC- cells in human breast cancer cell lines. Shown above are data from MCF7, T47D, and MDA-MB-231 lines. (A) MUC negative (-) and MUC+ cell sorts were cultured for up to 6 days. At two day intervals cells were counted using a hemacytometer. (B) Sorted fractions were labeled with BrdU for 1 hour prior to visualization of BrdU positive cells using anti-BrdU antibody. Data is reported as percentage of cells that incorporated BrdU. Error bars indicate SEM.

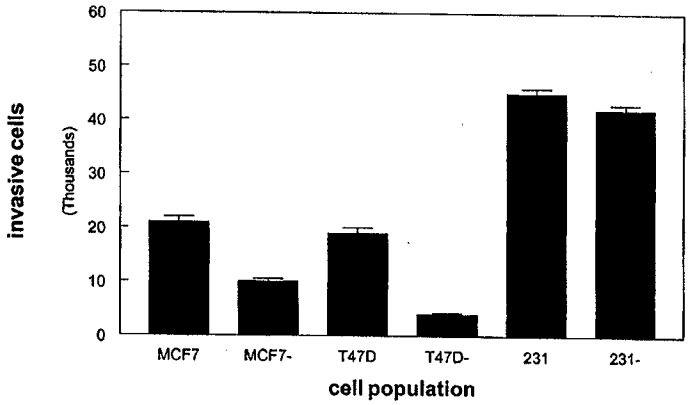


Fig. 3. Decreased invasive ability of MUC negative (-) population in MCF7 and T47D cell lines. MUC+ and MUC- cells from T47D, MCF7, and MDA-MB-231 lines were plated into Matrigel invasion chambers for 24 hours. Cells which invaded through the reconstituted basement membrane were fixed, stained, and counted. Error bars indicate SEM.

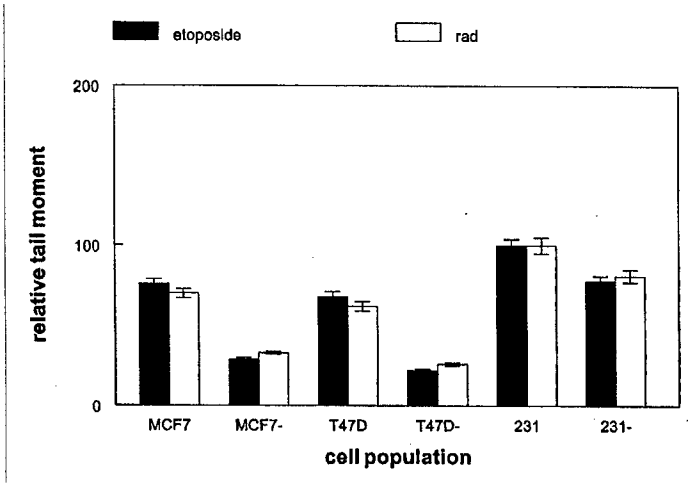


Fig. 4. Decreased DNA damage in the MUC negative (-) fraction after exposure to 10 μg/ml etoposide (filled bars) or 20 Gy ionizing radiation (open bars). MUC+ and MUC- cells from T47D, MCF7, and MDA-MB-231 lines were plated on tissue culture plastic dishes and subjected individually to one of the DNA damage protocols. Cells were then subjected to single cell gel electrophoresis. The extent of DNA damage (relative tail moment) was calculated based on signal intensity and migration. Error bars indicate SEM.

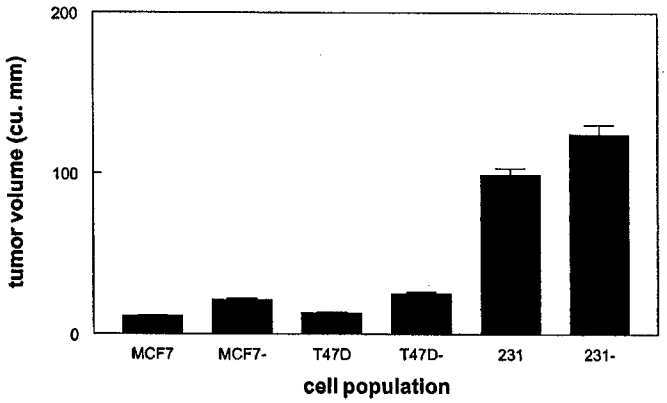


Fig. 5. The MUC- fraction (-) is more tumorigenic than the MUC+ fraction in human breast cancer cell lines. MUC+ and MUC- cells from T47D, MCF7, and MDA-MB-231 lines were injected into the mammary fat pads of nude mice. The tumor volume after one month was calculated from measurement of dissected tumors. Error bars indicate SEM.

Materials and Methods

Cell Culture and Sorting. Human breast cancer cell lines were cultured in Dulbecco's modified Eagle medium, 10% fetal bovine serum, and 40 μg/ml gentamicin. Double immunofluorescence labeling was performed by incubation of suspended cells with mouse anti-human epithelial specific antigen antibody and goat anti-human sialomucin antibody (20 μl/10^6 cells) at 4^0 C for 2 hours. After extensive washing in PBS, fluorescein conjugated anti-mouse IgG and rhodamine conjugated anti-goat IgG secondary antibodies were incubated with the cells for an additional two hours. After washing in PBS, cells were sorted using a Becton Dickinson flow cytometer.

Western Blot. 25 μg total cellular protein was separated by SDS-PAGE on 10% resolving gels under denaturing and reducing conditions. Separated proteins were electroblotted to PVDF membranes according to manufacturer's recommendations (Roche Molecular Biochemicals). Blots were incubated with antibodies to human smooth muscle actin, keratin 18, or keratin 19 (Sigma) for 16 hours at 4^0 C. After washing in Tris buffered saline containing 0.1% Tween 20 (TBST, pH 7.4), blots were incubated for 30 minutes at room temperature with anti-IgG secondary antibody conjugated to horseradish peroxidase. Following extensive washing in TBST, bands were visualized by the enhanced chemiluminescence method (Roche Molecular Biochemicals).

Cell Growth and Bromodeoxyuridine Incorporation Analysis. 3 x 10^4 sorted cells were cultured on tissue culture plastic dishes for up to six days. At two day intervals, cultures were trypsinized and counted using a hemacytometer. Duplicate cultures were incubated with 10 μM BrdU for 1 hour. After washing in PBS, cells were fixed in 70% ethanol, 50 mM glycine (pH 2) for 30 minutes at -20^0 C. After extensive washing in PBS, cells were incubated with mouse anti-BrdU primary antibody at 37^0 C for 30 minutes (Roche Molecular Biochemicals). After washing in PBS, cells were incubated with anti-mouse IgG secondary antibody conjugated to fluorescein at 37^0 C for 30 minutes. Following extensive washing in PBS, BrdU positive cells were visualized by fluorescence microscopy. The

number of positive cells was expressed as a percentage of total cells counted in ten randomly selected high power fields.

In Vitro Invasion Assays. 2 x 10^5 sorted cells were plated into modified Boyden chambers coated with Matrigel reconstituted basement membrane (Becton Dickinson) for 24 hours. Cells that were able to invade the semi-permeable membrane were fixed in methanol, stained with hematoxylin, and counted.

DNA Damage Analysis. 3 x 10^4 sorted cells were plated onto tissue culture plastic dishes for 24 hours before being treated with 10 μg/ml etoposide for 24 hours or exposed to 20 Gy ionizing radiation. Single cell gel electrophoresis then was performed following the manufacturer's instructions (Kinetic Imaging). Quantitation of DNA damage (relative tail moment) was performed using the manufacturer's image analysis software.

In Vivo Tumorigenesis. 2 x 10^5 sorted cells were injected subcutaneously into the mammary fat pads of nude mice. After one month, the fat pads were dissected and any tumors that developed were measured. Tumor volume was calculated as the product of the measurements in three axes.

KEY RESEARCH ACCOMPLISHMENTS

Task 1

All ESA+/MUC- populations proliferated 10-20% slower than the MUC+ cells; however the MUC- fraction in MCF7 and T47D lines grew 25-30% slower.

The MUC- fraction from MCF7 and T47D lines were 50-80% less able to penetrate reconstituted basement membrane (in vitro invasion assay). The MUC- fraction from 4 other breast cancer cell lines were highly invasive in the in vitro assay.

Task 2

The MUC- population from MCF7 and T47D cells exhibited 60% less DNA damage in the single cell gel electrophoresis analysis than the sorted fraction from 4 other breast cancer cell lines.

The MUC- population in all lines was more resistant to DNA damage than the MUC+ fraction.

Task 3

The MCF7 and T47D MUC- population were 90% less tumorigenic when injected into the mammary fat pads of nude mice than the same fraction from the other 4 cell lines.

The MUC- populations were more tumorigenic than the MUC+ fractions.

REPORTABLE OUTCOMES

Abstract

Crowe DL. 2005. Characterization of putative breast cancer stem cells. Deparment of Defense Breast Cancer Research Program Proceedings, p 29.

CONCLUSIONS

MUC- cells exist in human breast cancer cell lines with phenotypic properties different than those of the MUC+ population. The numbers of these cells vary widely and their properties are variable depending on the line from which they were derived. The cells expressed additional markers of epithelial and myoepithelial differentiation. The cells were difficult to maintain in culture for extended time periods due to generation of MUC+ progeny. Therefore we used short term assays to characterize these populations. All ESA+/MUC- populations proliferated slower than the MUC+ cells; however, the MUC- fraction in MCF7 and T47D lines grew even slower than those found in other lines. While the MUC- fraction in 4 other breast cancer cell lines were highly invasive in the in vitro assay, this subpopulation in MCF7 and T47D were less able to penetrate reconstituted basement membrane. The MUC- population from MCF7 and T47D cells showed less DNA damage than the sorted fraction from 4 other lines. The MUC- population in these lines generally was more resistant to DNA damage than the MUC+ fraction. The MCF7 and T47D MUC- population were also less tumorigenic when injected into mammary fat pads of nude mice. All MUC- populations were more tumorigenic than the MUC+ fractions. The MUC- fraction from different cell lines demonstrated putative stem cell characteristics to variable extents. Currently we are defining culture conditions which will allow long term maintenance of MUC- cells. We have also begun genetic characterization of the MUC- cells to determine those genes responsible for stem cell-like behavior. The results of the study and some of our new work on putative breast cancer stem cells was presented at the Department of Defense Breast Cancer Research Program meeting in Philadelphia, PA in June 2005.

REFERENCES

Blau HM, Brazelton TR, Weimann JM. 2001. The evolving concept of a stem cell: entity or function? Cell 105:829-841

Gudjonsson T, Villadsen R, Nielsen HL, Ronnov-Jessen L, Bissell MJ, Petersen OW. 2002. Isolation, immortalization, and characterization of a human breast epithelial cell line with stem cell properties. Genes Dev 16:693-706

Reya T, Morrison SJ, Clarke MF, Weissman IL. 2001. Stem cells, cancer, and cancer stem cells. Nature 414:105-111